Both Cell Fusion and Transdifferentiation Account for the Transformation of Human Peripheral Blood CD34-Positive Cells Into Cardiomyocytes In Vivo

Sui Zhang, MD, PhD; Dachun Wang, MD; Zeev Estrov, MD; Sean Raj; James T. Willerson, MD; Edward T.H. Yeh, MD

Background—Adult human peripheral blood CD34-positive (CD34⁺) cells appear to transform into cardiomyocytes in the injured hearts of severe combined immunodeficient mice. It remains unclear, however, whether the apparent transformation is the result of transdifferentiation of the donor stem cells or of fusion of the donor cell with the cardiomyocyte of the recipients.

Methods and Results—We performed flow cytometry analyses of cells isolated from the hearts of mice that received human CD34⁺ cells. Human HLA-ABC antigen and cardiac troponin T or Nkx2.5 were used as markers for cardiomyocytes derived from human CD34⁺ cells, and HLA-ABC and VE-cadherin were used to identify the transformed endothelial cells. The double-positive cells were collected and interphase fluorescence in situ hybridization was used to detect the expression of human and mouse X chromosomes in these cells. We found that 73.3% of nuclei derived from HLA⁺ and troponin T⁺ or Nkx2.5⁺ cardiomyocytes contain both human and mouse X chromosomes and 23.7% contain only human X chromosome. In contrast, the nuclei of HLA⁺, troponin T⁺ cells contain only mouse X chromosomes. Furthermore, 97.3% of endothelial cells derived from CD34⁺ cells contained human X chromosome only.

Conclusions—Thus, both cell fusion and transdifferentiation may account for the transformation of peripheral blood CD34⁺ cells into cardiomyocytes in vivo. (Circulation. 2004;110:3803-3807.)

Key Words: antigens, CD34 stem cell cardiomyocyte cell fusion

Stem cells of different origins have been observed to develop into a variety of cell types including cardiomyocytes,¹ hepatocytes, and epithelial cells of the gastrointestinal tract² after transplantation. The nature of this transformation, however, is unclear. Different research groups, using different detection methods in different experimental settings, have proposed different mechanisms for the apparent transformation of stem cells into cells of a variety of tissues.³⁻⁶ Some investigators attribute this transformation to the transdifferentiation potential of stem cells.¹²,⁶,⁷ Others have demonstrated that this apparent transformation is a result of cell fusion.³,⁴,⁸⁻¹⁰ It is widely known that stem cells of various origins can develop into cardiomyocytes. Many investigators have demonstrated that bone marrow hematopoietic stem cells, embryonic stem cells, adult mesenchymal stem cells,¹ hematopoietic stem cells in peripheral blood,¹¹,¹² cardiac progenitor cells,¹³ and adult cardiac stem cells¹⁴ home to the heart and transform into cardiomyocytes in vitro and in vivo. Most investigators attribute the phenotypic conversion to transdifferentiation.¹²,¹³,¹⁴ Recently, one report demonstrated that cell fusion¹³ is responsible for the transformation. We previously reported that human peripheral blood CD34-positive (CD34⁺) cells may develop into cardiomyocytes, vascular smooth muscle cells, and endothelial cells in the injured hearts of severe combined immunodeficient (SCID) mice.¹¹ Because the mechanism of the phenotypic transformation is important in evaluating the clinical feasibility of potential cellular therapy for heart failure, we have addressed the question of whether transformation of the CD34⁺ cells into cardiomyocytes is the result of transdifferentiation of the injected stem cells or fusion between donor cells and the cardiomyocytes of the recipient.

Methods

Animals
Female SCID mice (C3H, Jackson Laboratory, Bar Harbor, Maine) weighing 14 to 18 g were used in the study. The Institutional Animal Care and Use Committees of the University of Texas Health Science Center approved the study. Mice were randomly assigned to two experimental groups: one group received human CD34⁺ cells by intravenous infusion and the other group served as untreated controls. Mice were sacrificed 14 days after cell transplantation, and hearts were collected for further analysis.

Received June 2, 2004; revision received September 1, 2004; accepted September 21, 2004.

From the Departments of Cardiology (S.Z., S.R., E.T.H.Y.) and Bioimmunotherapy (Z.E.), University of Texas-M.D. Anderson Cancer Center; the Research Center for Cardiovascular Diseases, Institute of Molecular Medicine for the Prevention of Human Diseases, University of Texas-Houston Health Science Center (D.W., J.T.W., E.T.H.Y.); and the Texas Heart Institute/St. Luke’s Episcopal Hospital (J.T.W., E.T.H.Y.), Houston, Tex.

Guest Editor for this article was Robert A. Kloner, MD, PhD, University of Southern California Keck School of Medicine.

Correspondence to Edward T.H. Yeh, MD, University of Texas-M.D. Anderson Cancer Center, Faculty Bldg, Room 2036, 3rd Floor, Box 449, 1515 Holcombe Blvd, Houston, TX 77030. E-mail etyeh@mdanderson.org

© 2004 American Heart Association, Inc.

Circulation is available at http://www.circulationaha.org DOI: 10.1161/01.CIR.0000150796.18473.8E

3803
Peripheral Blood pore size of 70 time. The isolated cells were filtered through a cell strainer with a tissues were then incubated repeatedly at 37°C for 15 minutes each Worthington Biochemical), and pancreatin (0.6 mg/mL, Sigma). The enzymatic solution, ADS buffer containing elastase (0.3 mg/mL, Worthington Biochemical), type II collagenase (0.21 mg/mL, Worthington Biochemical), and stored at 4°C overnight before fluorescence-activated cell sorter (FACS) analysis. Cytoperm kit (BD Biosciences Pharmingen), and stored at 4°C previously described.11,15

Tissue Harvesting and Cell Isolation

The hearts were harvested 60 days after cell transplantation or MI (control animals). A published method16 was modified slightly and used to isolate cells from the heart. Briefly, the heart was cut into 6 pieces and placed in a 10-mL beaker containing 2 mL of the enzymatic solution, ADS buffer containing elastase (0.3 mg/mL, Worthington Biochemical), type II collagenase (0.21 mg/mL, Worthington Biochemical), and pancreatic (0.6 mg/mL, Sigma). The tissues were then incubated repeatedly at 37°C for 15 minutes each time. The isolated cells were filtered through a cell strainer with a pore size of 70 μm, fixed (20 minutes at 4°C) with the Cytofix/ Cytoperm kit (BD Biosciences Pharmingen), and stored at 4°C overnight before fluorescence-activated cell sorter (FACS) analysis.

Flow Cytometry Detection and Cell Sorting

The filtered cells were permeabilized with the Cytoperm wash kit (BD Biosciences Pharmingen) for 20 minutes at 4°C and incubated with a monoclonal antibody against cardiac troponin T (1:200, clone 1A11, Advanced ImmunoChemical, Long Beach, Calif) or with nonspecific mouse IgG-2, for 30 minutes at 4°C. After 3 washes, the cells were incubated with goat anti-mouse IgG (1:1000) conjugated with Alexa Fluor 488 (Molecular Probes) for 30 minutes at 4°C. In a separate set of experiments, mouse heart cells were incubated with a polyclonal antibody (goat, Santa Cruz Biotechnology, Santa Cruz, Calif) against a cardiac-specific transcription factor, Nkx2.5, or with nonspecific goat IgG for 30 minutes at 4°C. Cells were washed and incubated with chicken anti-goat IgG (1:1000) conjugated with Alexa Fluor 488 for 30 minutes at 4°C. After 3 washes, cells incubated with anti–cardiac troponin or with anti-Nkx2.5 were incubated again with phycoerythrin (PE)-conjugated anti-human HLA-ABC (clone W6/32, Cedarlane Laboratories, Hornby, Ontario, Canada) or PE-conjugated mouse IgG-2, for 30 minutes. For endothelial cell detection, isolated cells were incubated first with anti–vascular endothelium (VE)–cadherin (1:100, Bender MedSystems, Calif), then with the secondary antibody conjugated with Alexa Fluor 488. Cells were analyzed and sorted on a FACS/Aria flow cytometer (BD Biosciences). Gates were established by nonspecific immunoglobulin binding in each experiment. Approximately 30% to 40% of the entire population was sorted for the double-positive cells.

Polymerase Chain Reaction Analysis of Sorted Cells

Genomic DNA was isolated from heart cells sorted by expression of cardiac troponin T or by dual expression of human HLA-ABC and cardiac troponin T and was used to detect HLA expression by polymerase chain reaction (PCR). PCR analysis was performed with the primers derived from HLA-B exon 5–7 with the Advantage cDNA PCR kit (BD Biosciences Clontech). The following touchdown PCR program was used: 3 minutes at 94°C; 5 cycles of 30 seconds each at 94°C, 40 seconds each at 66°C, and 50 seconds each at 72°C; 3 cycles of 30 seconds each at 94°C, 40 seconds each at 64°C, and 50 seconds each at 72°C; and 1 cycle of 5 minutes at 72°C. The PCR products were analyzed by electrophoresis of 1% agarose gel, and the size of PCR amplicon derived from the HLA-B gene was ~280 bp.

Interphase Fluorescence In Situ Hybridization

The collected double-positive cells were spun onto a slide and fixed immediately with 3:1 methanol:acetic acid solution for 30 minutes. To quench the residual fluorescence from cell sorting, the slides were exposed to white light for 120 hours at 4°C. Complete diminishment of the residual fluorescence was confirmed by examination under an epifluorescence microscope (Nikon Eclipse TE2000U). Slides were briefly fixed in 3:1 methanol:acetic acid again and were predenatured, dehydrated, and denatured according to the manufacturer’s protocol. Slides were hybridized with a fluorescein isothiocyanate– conjugated DNA probe for mouse X chromosomes (ID Labs) and a PE-conjugated probe for human X chromosomes (Qbiogene) overnight at 37°C in a humidified chamber. After posthybridization wash, slides were counterstained with 4′,6-diamidino-2-phenyldinole (0.02 μg/mL) and examined with an epifluorescence microscope (Nikon Eclipse TE2000U).

Results

Engraftment of Human CD34+ Cells in the Heart

To evaluate engraftment of the CD34+ cells in the heart and transformation of these cells into the cardiomyocytes, we examined isolated cells by FACS analysis using specific antibodies against HLA-ABC, a surface marker for human cells, and cardiac troponin T, a cardiomyocyte-specific marker. HLA+ cells were detected in all 4 mice examined. Approximately 2% (2.0±0.4%) of the total cells from the heart were human HLA+ (Figure 1A), whereas cells from control mice with induced MI but not injected with CD34+ cells, were all HLA− (data not shown). FACS analysis of heart cells double-stained with antibodies against HLA and cardiac troponin T (Figure 1B) and with antibodies against HLA and Nkx2.5 (Figure 1C) demonstrated that ~1% of cells were double-positive, suggesting that these cardiomyocytes originated from the transplanted human cells. To ensure that immunostaining accurately reflects the genotype of isolated cells, DNA from the sorted double-positive cells and the cells stained only with anti–cardiac troponin was extracted for PCR detection of the human HLA-B gene. HLA-B fragment was amplified only in double-positive cells (Figure 1D). Thus, it is clear that the double-positive cells are of human origin.

Interphase Fluorescence In Situ Hybridization Analysis of Cardiomyocytes Developed From Transplanted CD34+ Cells

The population of double-positive cells was collected by cell sorting and examined with fluorescence in situ hybridization (FISH) analysis, in which specific probes for human and mouse X chromosomes were used simultaneously. The specificity of the probes was tested in mouse heart cells and human Hela cells by incubating these cells with both probes, and we confirmed that these 2 probes did not cross-react (Figure 2A). In the nuclei derived from cells that were troponin T+ but HLA−, only mouse X chromosomes were detected (Figure 2B). Because the recipient mice were female, 2 X chromosomes were observed in each nucleus. In troponin T+ and HLA+ cells, ~70% of the nuclei contained both human and mouse X chromosomes (Table 1, Figure 2C), suggesting that cell fusion had occurred. Because the human
donor is male, 1 human X chromosome was paired with 2 mouse X chromosomes in each nucleus (Figure 2C); however, \(\approx 30\%\) of the nuclei of troponin T\(^+\) cells contained only human X chromosomes (Table 1, Figure 2D), suggesting that transdifferentiation of CD34\(^+\) cells has also taken place. Analysis was also performed on HLA and Nkx2.5 sorted cells in 3 mice and similar findings were obtained (Table 1); however, only human X chromosomes were detected in \(\approx 97\%\) of cells stained positive to both anti–HLA-ABC and anti–VE-cadherin (Table 2).

Discussion

Different research groups have reported contradictory results on the nature of the apparent transformation of stem cells into cardiomyocytes. The discrepancy could have resulted from differences in the sources of stem cells, the approaches used to distinguish fusion from transdifferentiation, and the experimental settings.

In our study, we used an animal model in which SCID mice were transplanted with human peripheral blood CD34\(^+\) cells after the mouse hearts were injured by experimental MI. The specific antigen (human HLA-ABC) of the donor cells allowed us to track these cells accurately and rapidly using immunodetection methods, such as FACS analysis. Instead of evaluating tissue sections of the heart, we were able to examine quantitatively the entire heart and collect populations of cells of interest by using FACS sorting. Our approach, therefore, enables us to focus on an individual cell.
population of the heart and to perform further analysis, such as FISH, on the population.

Alvarez-Dolado et al\(^ {10} \) reported that cell fusion in a mouse model was responsible for transformation of bone marrow–derived cells into hepatocytes, Purkinje neurons, and cardiomyocytes. No transdifferentiation was observed in their study. Beltrami et al\(^ {14} \) demonstrated that adult cardiac stem cells differentiated into mature cardiomyocytes by transdifferentiation. Our results indicated that both cell fusion (73.3%\(^ {1} \)) and transdifferentiation (23.7%)\(^ {2} \) were involved in the transformation of transplanted stem cells into cardiomyocytes. The discrepancies between our results and those from the above 2 research groups could come from differences in the presence or absence of heart injury. The animals used in the study by Alvarez-Dolado et al were uninjured,\(^ {10} \) and the frequency of transdifferentiation was expected to be extremely low\(^ {2} \); alternatively, the results might relate to the number of cells analyzed. Both the Beltrami and Alvarez-Dolado groups analyzed tissue sections. In the study by Alvarez-Dolado and colleagues, 25 heart sections of a total thickness of 0.25 to 1.25 mm, which represented only a small portion of the heart, were analyzed.\(^ {10} \) The methods used to detect cell fusion might explain the differences in study results. Alvarez-Dolado’s team used a method based on Cre/lox recombination to detect fusion.\(^ {10} \) Beltrami et al\(^ {14} \) excluded the existence of cell fusion based on the number, location, size, and DNA content of the transplanted cells. In our study, the detection method was highly specific because we used FISH to detect specific human X chromosomes in a population of human cell–derived cardiomyocytes selected by FACS. Finally, the sources of transplanted stem cells could explain the differences that were found. In Alvarez-Dolado and colleagues’ study, hematopoietic cells from the bone marrow transformed into cardiomyocytes via fusion. Beltrami et al used adult cardiac stem cells. We used human peripheral blood CD34\(^ + \) cells. It is unclear, however, whether stem cells from different origins convert into the cells of recipient phenotype through different mechanisms. Oh et al have reported both fusion and transdifferentiation in a mouse model of ischemia reperfusion injury followed by the injection of Sca-1\(^ + \) cells.\(^ {13} \) Our experimental approach is closer to that of Oh et al and our results also are similar to theirs. We found that 97.3% of the endothelial cells derived from human CD34\(^ + \) cells contained only human X chromosomes. This is not surprising because the CD34\(^ + \) cell population contains endothelial progenitors that can differentiate into the mature endothelial cells naturally. Two groups have reported failures to observe transformation of bone marrow stem cells into cardiomyocytes in animal models.\(^ {17,18} \) The reason for their inability to detect cardiomyocyte transformation is not clear, but it could be related to differences in cell origin, cell preparation, and detection methodology.

In conclusion, our results suggest that human peripheral blood CD34\(^ + \) cells develop into cardiomyocytes in the injured hearts of SCID mice through both cell fusion and transdifferentiation. A number of groups have already embarked on the application of human bone marrow mesenchymal stem cells to repair damaged myocardium.\(^ {19–21} \) Our studies, thus, provide mechanistic insight on how human stem cells can transform into cardiomyocytes in vivo.

### References


### Table 1. FISH Analysis of Nuclei From Cells Double-Stained With Anti–HLA and Anti–Cardiac Troponin T or Anti–HLA and Anti–Nkx2.5 Antibodies in 7 Transplanted Mice

<table>
<thead>
<tr>
<th>X Chromosome in Nuclei</th>
<th>Human and Mouse</th>
<th>Human</th>
<th>Mouse</th>
<th>Total Nuclei Counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA(^ + ) and troponin(^ + ), n (%)</td>
<td>70 (70)</td>
<td>28 (28)</td>
<td>2 (2.0)</td>
<td>100</td>
</tr>
<tr>
<td>HLA(^ + ) and Nkx2.5(^ + ), n (%)</td>
<td>84 (71.2)</td>
<td>31 (26.3)</td>
<td>3 (2.5)</td>
<td>118</td>
</tr>
<tr>
<td>Mouse 1, n (%)</td>
<td>96 (78.7)</td>
<td>24 (19.7)</td>
<td>2 (1.6)</td>
<td>122</td>
</tr>
<tr>
<td>Mouse 2, n (%)</td>
<td>91 (71.1)</td>
<td>36 (28.1)</td>
<td>1 (0.8)</td>
<td>128</td>
</tr>
<tr>
<td>Mouse 3, n (%)</td>
<td>91 (78.4)</td>
<td>19 (16.4)</td>
<td>6 (5.2)</td>
<td>116</td>
</tr>
<tr>
<td>Mouse 4, n (%)</td>
<td>47 (69.1)</td>
<td>17 (25)</td>
<td>4 (5.9)</td>
<td>68</td>
</tr>
<tr>
<td>Mouse 5, n (%)</td>
<td>86 (74.8)</td>
<td>26 (22.6)</td>
<td>3 (2.6)</td>
<td>115</td>
</tr>
<tr>
<td>Mean±SE</td>
<td>73.3±1.5%</td>
<td>23.7±1.7%</td>
<td>2.9±0.7%</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. FISH Analysis of Nuclei From Cells Double-Stained With Anti–HLA-ABC and Anti–VE-Cadherin Mice Transplanted With Human Peripheral Blood CD34\(^ + \) Cells

<table>
<thead>
<tr>
<th>X Chromosome in Nuclei</th>
<th>Human and VE-Cadherin(^ + )</th>
<th>Human</th>
<th>Mouse</th>
<th>Total Nuclei Counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse 1, n (%)</td>
<td>1 (1)</td>
<td>101 (97.1)</td>
<td>2 (1.9)</td>
<td>104</td>
</tr>
<tr>
<td>Mouse 2, n (%)</td>
<td>0 (0)</td>
<td>127 (97.7)</td>
<td>3 (2.3)</td>
<td>130</td>
</tr>
<tr>
<td>Mouse 3, n (%)</td>
<td>1 (0.8)</td>
<td>119 (98.4)</td>
<td>1 (0.8)</td>
<td>121</td>
</tr>
<tr>
<td>Mouse 4, n (%)</td>
<td>0 (0)</td>
<td>136 (99.3)</td>
<td>1 (0.7)</td>
<td>137</td>
</tr>
<tr>
<td>Mouse 5, n (%)</td>
<td>5 (3.6)</td>
<td>131 (95)</td>
<td>2 (1.4)</td>
<td>138</td>
</tr>
<tr>
<td>Mouse 6, n (%)</td>
<td>3 (2)</td>
<td>141 (96)</td>
<td>3 (2)</td>
<td>147</td>
</tr>
<tr>
<td>Mean±SE</td>
<td>1.2±0.6%</td>
<td>97.3±0.6%</td>
<td>1.5±0.2%</td>
<td></td>
</tr>
</tbody>
</table>


Both Cell Fusion and Transdifferentiation Account for the Transformation of Human Peripheral Blood CD34-Positive Cells Into Cardiomyocytes In Vivo
Sui Zhang, Dachun Wang, Zeev Estrov, Sean Raj, James T. Willerson and Edward T.H. Yeh

_Circulation_. 2004;110:3803-3807; originally published online December 13, 2004; doi: 10.1161/01.CIR.0000150796.18473.8E

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/110/25/3803

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/